Egypt. Acad. J. biolog. Sci., 2 (2): 1- 4 (2010)
Email: egyptianacademic@yahoo.com
Received: 29/5/2010

F. Toxicology &Pest control
ISSN: 2090 - 0791
www.eajbs.eg.net

Evaluation of cytogenetic effects on bovine peripheral lymphocytes after the treatment with tebuconazole

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ABSTRACT

Tebuconazole-based fungicide (group of triazole fungicides, with 25% of active agent) was evaluated for their ability to induce sister chromatid exchange (SCE) and proliferation indices (PI) in cultured bovine peripheral lymphocytes. The cultures were treated with the fungicides at the concentrations ranged from 3 to 60 $\mu g.ml^{-1}$ for the last 24 and 48 h of incubation. Positive results in the SCE induction were obtained after the exposure to tebuconazole for the last 24h of incubation. The highest concentration of the fungicide also reflected in reduction of proliferation activity, but only insufficient number of cells could be analysed. Our results correspond to our previous finding from the CA assay (chromosomal aberrations) in bovine peripheral lymphocytes treated with the fungicide. Prolonged time of exposure provided inadequate evidence for the genotoxic activity of the fungicide.

Key words: tebuconazole-based fungicide, bovine peripheral lymphocytes, sister chromatid exchanges

INTRODUCTION

Conazoles are a large family of synthetic fungicides used extensively in agricultural applications. Conazole fungicides containing triazoles as active ingredients (e.g., myclobutanil, propiconazole, and triadimefon) are the most effective agents on fruit, vegetables, cereals, and seeds protection against Fusarium species at present. On the basis of the results of experimental studies efficacies of triazole fungicides ranged from 25% to 89% (Haidukowski *et al*. 2005) and depended on the time of application. Several conazole compounds were also used pharmaceutically, primarily for the treatment of candidiasis, cryptococcosis, and coccidiomycosis (Rockett *et al*. 2006).

Tebuconazole is efficient against broad spectra of fungous diseases. It belongs to the most widely used triazole fungicide in the European Union. Fungicide mode of action of triazoles is realised by inhibition the cytochrome P450 (CYP)-51 by the way of inhibition of 14a demethylase, enzyme that is essential for biosynthesis of ergosterol (Klix *et al*. 2007). Besides this enzyme, triazoles also modulate the expression and function of multiple CYP and other metabolic enzymes in the mammalian liver. Changes in the expression and activity of a number of CYP enzymes after the exposure of rodents to conazole fungicides are resulted in multiple toxic endpoints, including carcinogenic, neurological, reproductive, and endocrinological effects (Sun *et al*. 2005, 2006).

Damage in DNA structure may be critical step in a process of carcinogenesis. General cytogenetic biomarkers used in the assessment of genotoxic effects of chemical agents include chromosomal aberrations (CA), sister chromatid exchanges (SCE) and micronuclei (MN) frequency (Remor *et al*. 2009). Positive genotoxic effects were described for atrazine and paraquat (Ribas *et al*., 1995) in human peripheral lymphocytes. Permetrin (Undeger and Basaran 2005) and deltametrin (Chauhan *et al*. 2007) were genotoxic in mammalian test systems *in vivo* and *in vitro*. A wide spectrum of chromosome damage were described by Yilmaz *et al*. (2008) in mouse bone

marrow cells and human lymphocytes fungicide after the exposure to Conan 5FL (containing 50 g/l hexaconazole). This fungicide caused structural and numerical abnormalities in both mammalian cells; sister chromatid union, chromatid and chromosome breaks, fragments, dicentric and ring chromosomes, and polyploidy. Significant increases in the SCE frequency in a dose dependent manner were found in human lymphocytes.

The information on the genotoxic effects of pesticides in domestic animals is limited and inconsistent. Numerous chromosome lesions, CA, SCE and cytotoxic effects were found after exposure to the herbicide bifenox that belongs to the group of triazole pesticides in bovine peripheral lymphocytes *in vitro*. (Šiviková and Buleca 1999; Šiviková and Dianovský 1999).

In the present paper the early effects of tebuconazole-based fungicide on cultured bovine peripheral lymphocytes were investigated using SCEs as biomarkers. The SCE analysis is considered as a very sensitive indicator of genotoxicity, also the mechanism of their induction is still not known. Recent studies revealed the nucleotide pool imbalance can have severe consequences on DNA metabolism that is a critical point in the SCE formation (Bolognesi 2003).

MATERIAL AND METHODS

The tebuconazole-based fungicide, trade name Orius α- terc.butyl - α – (4-chlorophenylethyl) -1 H -1,2,4 - triazolyl-ethanol, 25% of active agent was soluble in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, USA) and applied into the culture flasks at concentrations of 3, 6, 15, 30 and 60 μg.ml^{-1}. Mitomycin C (MMC Sigma, St. Louis, MO, USA, 0.4 μmol.l^{-1}) was used as a positive control agent. Experiments were carried out on two healthy cow donors (Slovak spotted cattle, 6 months old).

Whole blood cultures (0.5 ml) were cultivated for 72 h at 38 °C in 5 ml of RPMI 1640 medium supplemented with L-glutamine, 15 μmol.ml^{-1} HEPES (Sigma, St. Louis, MO, USA), 15% foetal calf serum, antibiotics (penicillin 250 U/ml and streptomycin 250 μg.ml^{-1}), and phytohaemagglutinin (PHA, 180 μg.ml^{-1}, Welcome, Dartford, England). Slides were obtained using the standard cytogenetic method; 2 h before the harvest, colchicine (Merck, Darmstadt, Germany) was added at a concentration of 5 μg. ml^{-1}. Lymphocyte cultures were exposed to the tested fungicide for the last 24 h and 48 h of cultivation. For the SCE assay and the cell cycle kinetics, bromodeoxyuridine (8 μg.ml^{-1}, BrdUrd, Sigma, St. Louis, MO, USA) was added to all the cultures 24 h after initiation of division and slides were stained with FPG technique. Fifty differentially stained metaphases for the SCE and one hundred metaphases for determination of M_1, M_2 and M_{3+} mitotic divisions were examined. The statistical analysis of results was performed using a simple analysis of variance (ANOVA), and then the Student` s t test was applied to compare SCE occurrence between treated and untreated groups. A χ^2 test was employed to estimate the cell cycle delay.

RESULTS

The results obtained from the SCE studies and the lymphocyte proliferation kinetics are summarised in Tables 1 and 2.

A 24 h exposure to the tebuconazole-based fungicide showed statistically significant elevations in the mean of SCEs in each donor at the concentrations ranged from 15 to 60 μg.ml^{-1} ($p<0.05$ or $p<0.01$ and $p<0.001$, respectively by ANOVA and Student's t test).

Table 1: Frequency of SCEs and proliferation indices in cultured peripheral lymphocytes exposed to the fungicide tebuconazole for 24h

Dose	SCE / cell	PI
	Donor 1	
Control (DMSO)	6.58 ± 2.40	2.13
Fungicide tebuconazole (μg.ml^{-1})24 h		
3	6.94 ± 2.42^{a}	2.10^{a}
6	7.10 ± 2.87^{a}	2.14^{a}
15	7.90 ± 2.43**	1.99^{a}
30	9.42 ± 2.83 ***	2.00^{a}
60	10.83 ± 2.56^{***,b}	1.72^{***,c}
Positive control, 0.4 μM MMC	9.74 ± 2.76 ***	1.95 ^{a}
	Donor 2	
Control (DMSO)	6.30 ± 2.14	2.06
Fungicide tebuconazole (μg.ml^{-1})24 h		
3	6.60 ± 2.47^{a}	2.07^{a}
6	7.00 ± 2.59^{a}	2.17^{a}
15	7.56 ± 2.96*	2.16^{a}
30	9.24 ± 2.90***	1.93^{a}
60	9.82 ± 3.42^{***,d}	1.78^{***,e}
Positive control 0.4 μM, MMC	9.86 ± 2.30***	1.94^{a}

A total of 50 second - division metaphases of each group were analysed for SCE, if it was possible.

*, **, *** statistically significant data (P<0,05, P<0,01, P<0,001, ANOVA, Student`s t test)

a - no statistical significance, b, c –uncompleted number of cells, (29 and 50, respectively), d, e – uncompleted number of cells (24 and 38, respectively) .

The highest concentrations are reflected in the reduction of the PI in both donors (χ^2 test, $p<0.001$), but only insufficient number of cells could be analysed.

No dose dependence in elevations of SCE was obtained after the prolonged time of exposure to tebuconazole-based fungicide. After the treatment with the fungicide at a dose of 6 $\mu g.ml^{-1}$ for 48h, maximum frequency of SCEs but (without a statistical significance) was observed.

Table 2: Frequency of SCEs and proliferation indices in cultured peripheral lymphocytes exposed to the fungicide tebuconazole for 48h

Dose	SCE / cell	PI
	Donor 1	
Control (DMSO)	6.76 ± 2.94	1.84
Fungicide tebuconazole ($\mu g.ml^{-1}$)48 h		
3	6.96 ± 2.46 [a]	1.71 [**]
6	7.74 ± 3.02 [a]	1.72 [**]
15	7.42 ± 2.31 [a]	1.90 [a]
30	7.02 ± 2.20 [a]	1.82 [a]
60	ND	ND
Positive control, 0.4 μM MMC	23.2 ± 3.9 [***]	1.55 [*]
	Donor 2	
Control (DMSO)	6.70 ± 3.02	1.88
Fungicide tebuconazole ($\mu g.ml^{-1}$)48 h		
3	6.76 ± 2.67 [a]	1.85 [a]
6	7.54 ± 2.91 [a]	1.67 [***]
15	7.34 ± 2.08 [a]	1.87 [a]
30	6.84 ± 2.26 [a]	1.76 [**]
60	ND	ND
Positive control 0.4 μM, MMC	24.5 ± 4.8 [***]	1.44 [***]

A total of 50 second - division metaphases of each group were analysed for SCE, if it was possible.
, * statistically significant data (P<0,01, P<0,001, ANOVA, Student`s t test)
a-no statistical significance.
ND – not done

This concentration also revealed in statistically significant reduction of the PI (χ^2 test, $p<0.01$or $p<0.001$). In comparison to this dose, both highest concentrations (15 and 30 $\mu g.ml^{-1}$, respectively) showed a decrease in the frequency of SCEs. The former dose caused an improvement in the proliferation activity in both donors. A reduction in the PI frequencies with a statistically significance was seen after the application of the later dose in donor 2 ($p<0.01$, Table 2). The highest concentration (60 $\mu g.ml^{-1}$) caused a reduction of total cell number.

DISCUSSION

The tebuconazole-based fungicide tested in bovine peripheral lymphocytes was shown to be able to induce cellular lesions that lead to SCE formation and/or to the death of cells.

A significant effect of the fungicide on SCE increase was found in the treatment for 24 h at the concentrations ranged from 15 to 60 $\mu g.ml^{-1}$ (Student's t test). The maximum frequency of the exchange was achieved after the exposure to the highest doses (60 $\mu g.ml^{-1}$), but only insufficient number of cells could be analysed ($p<0.001$). A statistically significant reduction of cell proliferation (PI, $p<0.01$, χ^2 test) was found as well.

After the prolonged time of exposure inadequate results for confirmation of genotoxicity of the fungicide were obtained. A peak of replication damage but without a statistical significance was obtained after the exposure to the fungicide at a concentration of 6 $\mu g.ml^{-1}$ that was correlated with the cell cycle delays in both donors ($p<0.01$ or 0.001). When compared to this dose, both higher concentrations tested (15 and 30 $\mu g.ml^{-1}$) caused a decrease in the mean of SCEs. After the application of the former dose (15μg/ml) an improvement of the proliferation activity was occurred. Proliferation activity increase was probably caused by a decrease of more damaged cells (Morimoto 1984), and thus collections of less damage cells could be observed. Evaluation of proliferation indices represents an additional check on the chemical agent potency and reduction of the PI can be interpreted as cell cycle delays and/or in terms of induced cytotoxicity (Preston *et al*. 1987; Rojas *et al*. 1993). The cytotoxic effect of tebuconazole-based fungicide was seen after the application at the concentration of 3, 6, and in donor 2 also after the concentration of 30 $\mu g.ml^{-1}$. Consecutive treatments with the fungicide at the concentration of 60 $\mu g.ml^{-1}$caused total letality of cells.

The enumeration of SCE is the most widely used approach for the assessment of the genotoxic/cytogenetic effects of various chemical compounds. Our results correspond with several other authors who have regarded SCEs as very sensitive bioindicators of genotoxic agents (Cabalo *et al*. 1992; Tucker and Preston 1996). Similarly, Scarpato *et al*. (1996) reported

that highly damaged cells, which enter into mitoses, were delayed or failed to occur; this might mask the expression of genotoxic effects.

In conclusion, our results indicate that the tebuconazole-based fungicide is able to exert cytotoxic and genotoxic effects in bovine peripheral lymphocytes.

ACKNOWLEDGEMENTS

This work was supported by the Slovak Research and Development Agency under the contract no. APVV-0010-07, the Ministry of Education and Science of the Slovak Republic 1/0334/10 and NRL of UVM

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